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Coulson-Gilmer, C., Humphries, M. P., Sundara Rajan, S., Droop, A., Jackson, S., Condon, A., Cserni, G., Jordan, L. B., Jones, L. J., Kanthan, R., Di Benedetto, A., Mottolese, M., Provenzano, E., Kulka, J., Shaaban, A. M., Hanby, A. M., & Speirs, V. (2018). Stanniocalcin 2 expression is associated with a favourable outcome in male breast cancer. *The Journal of Pathology: Clinical Research*, 4(4), 241-249. <https://doi.org/10.1002/cjp2.106>

### **Published in:**

The Journal of Pathology: Clinical Research

### **Document Version:**

Publisher's PDF, also known as Version of record

### **Queen's University Belfast - Research Portal:**

[Link to publication record in Queen's University Belfast Research Portal](#)

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# Stanniocalcin 2 expression is associated with a favourable outcome in male breast cancer

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## Abstract

Breast cancer can occur in either gender; however, it is rare in men, accounting for <1% of diagnosed cases. In a previous transcriptomic screen of male breast cancer (MBC) and female breast cancer (FBC) occurrences, we observed that *Stanniocalcin 2* (*STC2*) was overexpressed in the former. The aim of this study was to confirm the expression of *STC2* in MBC and to investigate whether this had an impact on patient prognosis. Following an earlier transcriptomic screen, *STC2* gene expression was confirmed by RT-qPCR in matched MBC and FBC samples as well as in tumour-associated fibroblasts derived from each gender. Subsequently, *STC2* protein expression was examined immunohistochemically in tissue microarrays containing 477 MBC cases. Cumulative survival probabilities were calculated using the Kaplan–Meier method and multivariate survival analysis was performed using the Cox hazard model. Gender-specific *STC2* gene expression showed a 5.6-fold upregulation of *STC2* transcripts in MBC, also supported by data deposited in Oncomine™. *STC2* protein expression was a positive prognostic factor for disease-free survival (DFS; Log-rank; total  $p = 0.035$ , HR = 0.49; tumour cells  $p = 0.017$ , HR = 0.44; stroma  $p = 0.030$ , HR = 0.48) but had no significant impact on overall survival (Log-rank; total  $p = 0.23$ , HR = 0.71; tumour cells  $p = 0.069$ , HR = 0.59; stroma  $p = 0.650$ , HR = 0.87). Importantly, multivariate analysis adjusted for patient age at diagnosis, node staging, tumour size, ER, and PR status revealed that total *STC2* expression as well as expression in tumour cells was an independent prognostic factor for DFS (Cox regression;  $p = 0.018$ , HR = 0.983;  $p = 0.015$ , HR = 0.984, respectively). In conclusion, *STC2* expression is abundant in MBC where it is an independent prognostic factor for DFS.

**Keywords:** male breast cancer; stanniocalcin 2; immunohistochemistry; survival

Received 27 March 2018; Revised 30 May 2018; Accepted 25 June 2018

No conflicts of interest were declared.

## Introduction

Breast cancer (BC) is rare in men, accounting for <1% of diagnosed cases. Treatment is informed by clinical

trials conducted in women, however, recent literature suggests that, while similar histologically, there are differences in genomic profiles between genders, which may be exploited therapeutically [1–3].

In our efforts to define biological differences in male breast cancer (MBC) and female breast cancer (FBC), we have previously conducted gene expression analysis in matched MBC and FBC [3]. We observed that *Stanniocalcin 2* (*STC2*) was frequently overexpressed in MBC with indications that this gene showed the greatest fold change between genders. *STC2* was identified in 1998, cloned from a human osteosarcoma cDNA library and is related to a secreted glycoprotein found in bony fish, where it plays a role in calcium and phosphate homeostasis [4]. The *STC2* gene encodes a 302 amino acid protein, which shares 30–39% homology with its sister molecule *STC1* [4–6]. This 56 kDa secreted glycoprotein forms homodimers, and has putative roles in cell survival, dormancy, and metastasis. It has been suggested to function in an autocrine/paracrine manner [5–10].

*STC2* is expressed in many mammalian tissues, including kidney, pancreas, intestine, and liver [8,11]. In FBC, *STC2* is overexpressed compared to normal human breast tissue [12]. *STC2* is oestrogen responsive, is frequently co-expressed with ER [13,14] and is preferentially expressed in breast tumours of luminal phenotype [15]. It is overexpressed in other cancers, including lung [16], ovarian [17] as well as in colorectal and gastric cancer in which it is thought to play a role in cancer metastasis and progression [9,10]. However, in FBC, *STC2* expression appears to be a favourable prognostic factor, associated with extended disease-free and overall survival [15,18,19].

As *STC2* has not been examined in the context of MBC, the aim of this study was to validate our initial microarray findings, then investigate the expression of *STC2* on clinical outcome in a large cohort of MBCs by immunohistochemistry (IHC).

## Materials and methods

### Ethical approval and patient material

Leeds (East) Research Ethics Committee (06/Q1205/156; 15/YH/0025) granted ethical approval. Initial transcriptomics comparing genders used cases matched for age, size, nodal, and survival status, as described previously [3]. An additional three male and three female age-matched ER+, PR+, HER– ductal carcinomas (fresh-frozen) were used to confirm *STC2* gene expression. This was also performed on cultured fibroblasts derived from a further four male and three female samples of the same phenotype, prepared as previously described [20].

### Gender comparison of *STC2* gene expression

Gene expression data for male and female BCs was obtained using the Almac Breast Cancer DSA™ platform as described previously [3]. Microarray data are available on ArrayExpress (www.ebi.ac.uk/arrayexpress) with accession number E-MTAB-4040. The OncoPrint™ platform was used for further data mining. Transcriptomics data were confirmed using qRT-PCR, with reagents from Invitrogen unless otherwise stated. RNA was extracted from fresh-frozen breast tumours and cultured fibroblasts (RNeasy kit, Qiagen Cat #74106, Manchester, UK) according to manufacturer's instructions. Prior to cDNA synthesis, genomic DNA was removed using the TURBO DNA-free™ kit (#AM1907). Following 90 s centrifugation at 8000 × *g*, the supernatant was transferred to a fresh Eppendorf. Levels and quality of RNA were assessed using Nanodrop. RNA was then reverse transcribed: 1 µl Random hexamers (50 µM, Invitrogen #N8080127, Paisley, UK), 1 µl of 10 mM dNTP stock (#D7295, Sigma-Aldrich, Poole, UK) were added and incubated for 5 min at 65 °C, then placed on ice for 2 min. Remaining reagents were from SuperScript Reverse Transcriptase kit (Invitrogen #18064014) unless otherwise specified. Per sample, 4 µl 5× first strand buffer, 2 µl 0.1 M dithiothreitol and 1 µl RNase out (Invitrogen #10777019) were added and samples incubated for 5 min at room temperature, then for 2 min at 42 °C. Superscript II enzyme (1 µl) was added to each sample, then samples were heated at 42 °C for 50 min, followed by a 15 min incubation at 70 °C. Samples were placed on ice for 2 min, and cDNA concentration measured using Nanodrop.

For RT-qPCR, each well contained 90 ng cDNA, 10 µl TaqMan (Universal PCR) MasterMix (II), 1 µl primer (TaqMan, ×20 Thermo Fisher Scientific, Loughborough, UK #4331182; *STC1* (Hs00174970\_m1), *STC2* (Hs01063215\_m1), *RPLP0* (Hs99999902\_m1)) in a 20 µl reaction volume. cDNA was replaced with dH<sub>2</sub>O in negative controls.

Reactions were heated to 50 °C for 2 min then 90 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min using a QS5 PCR machine. All reactions were performed in triplicate. The mean values for the replicates for each sample were calculated and expressed as cycle threshold. Gene expression levels of *STC2* were expressed as  $2^{-\Delta\Delta C_t}$ , in which  $\Delta\Delta C_t$  was normalised to the  $C_t$  value of *RPLP0* (loading control) and to a calibrator sample when the assay ran across more than one plate.

### Immunohistochemistry

Levels of *STC2* were examined by IHC in 477 MBCs represented on tissue microarrays as described

previously [3]. REMARK criteria were employed [21] and patient characteristics are shown in Table 1. As the cases covered several tissue microarrays (TMAs), slides were batch stained for consistency. Slides were placed on a heat block for 20 min and then placed into 1× access revelation solution (Menarini, High Wycombe, UK), which was then heated to 125 °C for 2 min in a pressure cooker. Slides were transferred for 1 min to 90 °C automation wash buffer before being placed under running water for 1 min. Slides were transferred to TBS-T, then endogenous peroxidase activity was quenched by adding 2 drops of peroxidase block (Novocastra, Newcastle, UK, RE7101-CE) for 20 min. Slides were placed into TBS-Tween (0.1%) for 5 min. Sections were blocked with 1:10 Casein solution (Vector Laboratories® #SP-5020, Peterborough, UK) in antibody diluent (Thermo Fisher Scientific #003218) to block non-specific staining, then incubated overnight at 4 °C with STC2 antibody (manufacturer: Atlas antibodies, supplier: Cambridge Bioscience, Cambridge, UK, HPA045372) solution 1:400 in antibody diluent (isotype controls were diluted to the same

final concentration). Antibody specificity was confirmed by the manufacturer by Western blot, IHC, and immunofluorescence, validated by the Human Protein Atlas (<http://www.proteinatlas.org>) and has been used successfully in other published works [22]. We extended this by optimising the concentration using a multi-tissue block containing positive control tissue (human intestine and liver), and a matched isotype control was used to determine antibody specificity. TMAs were batch stained alongside the multi-tissue block as well as each TMA including its own positive control tissue (human intestine and liver). Slides were then washed three times in TBS-T (5 min each). Novocastra kit (Leica Biosystems, #RE7230-CE) was used for secondary staining according to manufacturer guidelines. Following incubation with DAB chromogen, slides were rinsed in 1× PBS (5 min) followed by running tap water (1 min). Slides were then counterstained with Mayer's haematoxylin, blued with Scotts tap water, then dehydrated and mounted with permanent aqueous medium DPX (Sigma-Aldrich). TMAs were digitised (×10 magnification, Leica-Aperio AT2 ScanScope scanner; Leica Biosystems, Newcastle, UK).

Each TMA core was viewed and scored using QuPath software [23]. In brief, TMAs were identified using the TMA dearrayer tool, and the TMA map imported. Tissue was detected using the 'simple tissue detection' tool, so that any whitespace was excluded from the analysis. Any confounding objects such as tissue folds were removed manually at this stage. Cells were detected using the 'cell detection' tool. Polygons were drawn around a total of 7500 cells across 6 separate TMAs, setting cell class as tumour or stroma. These 'training objects' were then used to create a detection classifier, which recognises a variety of cellular features to designate regions as tumour or stroma. The cells were then classified as + [>0.1], ++ [>0.25], +++ [>0.5], or negative [<0.1] (intensity cut-off points shown in square brackets). The detection classifier was run on all TMAs. STC2 expression was assessed quantitatively using the H-score [13,22]. The H-score takes into account both staining intensity and percentage of cells stained, giving a range of 0–300 using the following formula:  $1 \times (\% \text{ cells } +) + 2 \times (\% \text{ cells } ++)$  +  $3 \times (\% \text{ cells } +++)$ . Overall scores were averaged from duplicate or triplicate cores, which represented a case and a minimum of 200 tumour cells were evaluated.

## Statistical analysis

Unpaired two tailed *t*-tests were used for STC2 expression analysis. Receiver operating characteristic (ROC) curves [24] were generated for tumour and stroma

**Table 1.** Clinicopathological characteristics for the IHC cohort

Characteristics	
Mean age (range)	66 (30–97)
Mean follow-up, years (range)	3.9 (0.08–24.5)
Mean tumour size mm (range)	21.2 (1–86)
	Number (%)
Histology	
Invasive	419 (88)
DCIS	7 (1)
Mixed	15 (3)
Unknown	36 (8)
Grade	
1	50 (10)
2	193 (41)
3	147 (31)
Unknown	87 (18)
Lymph node status	
Positive	134 (28)
Negative	147 (31)
Unknown	196 (41)
ERα	
Positive	404 (85)
Negative	30 (6)
Unknown	43 (9)
PR	
Positive	352 (74)
Negative	74 (15)
Unknown	51 (11)
HER2	
Positive	6* (1)
Negative	291 (61)
Unknown	180 (38)

DCIS, ductal carcinoma *in situ*.

\*Confirmed by FISH/CISH.

cells using disease-free survival (DFS; from initial diagnosis to the diagnosis of local or distant recurrence), and used to determine clinically relevant cut-off points for STC2 H-scores. Univariate analysis was then performed: the STC2 H-score data were dichotomised using the identified STC2 cut-off points and associations with both DFS and overall survival (OS; from initial diagnosis to death) were analysed by Log-rank test. Multivariate analysis was also performed using the Cox proportional hazards regression model. Clinicopathological variables included in multivariate analysis were age at diagnosis, node staging, tumour size, ER, and PR status. Patients were censored at the last date they were known to be alive.

## Results

### Gene expression analysis

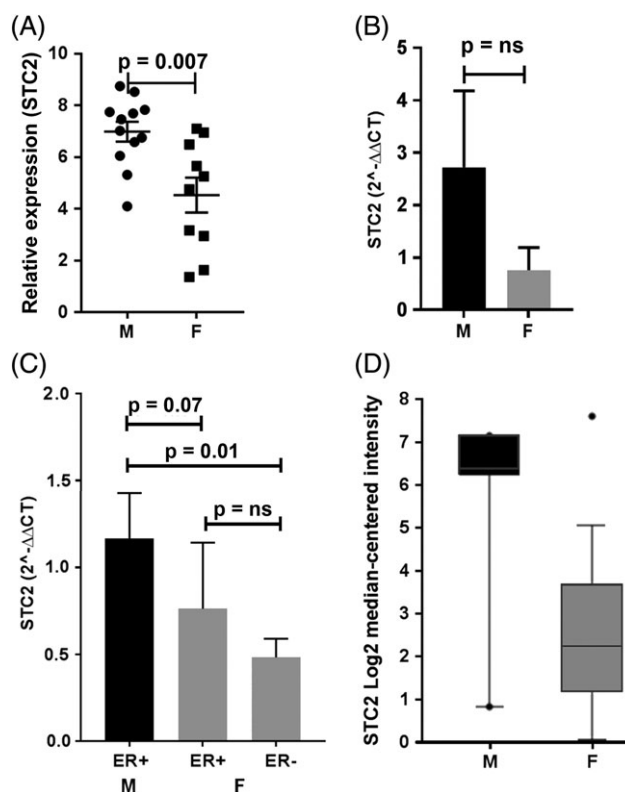
Comparing genders, we observed significant upregulation of *STC2* in MBC compared to FBC, with a mean fold-change of 5.61 (Figure 1A;  $p = 0.007$ ), with RT-qPCR of independent samples (3× male; 3× female) suggesting a similar trend (Figure 1B). While this did not reach statistical significance, higher expression was also seen using RT-qPCR of breast fibroblasts derived from a further four male and three female, age-matched ER+, PR+, HER– ductal carcinomas (Figure 1C) and confirmed by interrogating Oncomine™ (Figure 1D).

### STC2 IHC

STC2 staining was predominantly cytoplasmic with occasional foci of plasma membrane immunoreactivity. Representative images are shown in Figure 2A. All samples showed some tumour cell STC2 positivity, and similarly in the stroma weak staining was observed in the majority of cases. The breakdown of staining intensities in tumour and stroma is shown in Figure 2B. In addition, there was a significant positive correlation between STC2 H-scores in the tumour and stroma, (Spearman rank  $\rho = 0.929$ ,  $p < 0.001$ ; Pearson correlation  $R = 0.893$ ,  $p < 0.001$ ).

### Impact of STC2 expression on survival

Cut-offs for high total, tumour, and stroma STC2 immunoreactivity, defined by ROC curve analysis were  $>90.5$ ,  $>108.5$ , and  $>28.4$ , respectively (data not shown). By univariate analysis, high-total STC2 as well as in both tumour and stroma individually impacted on DFS but not OS (Figure 3). Cases with

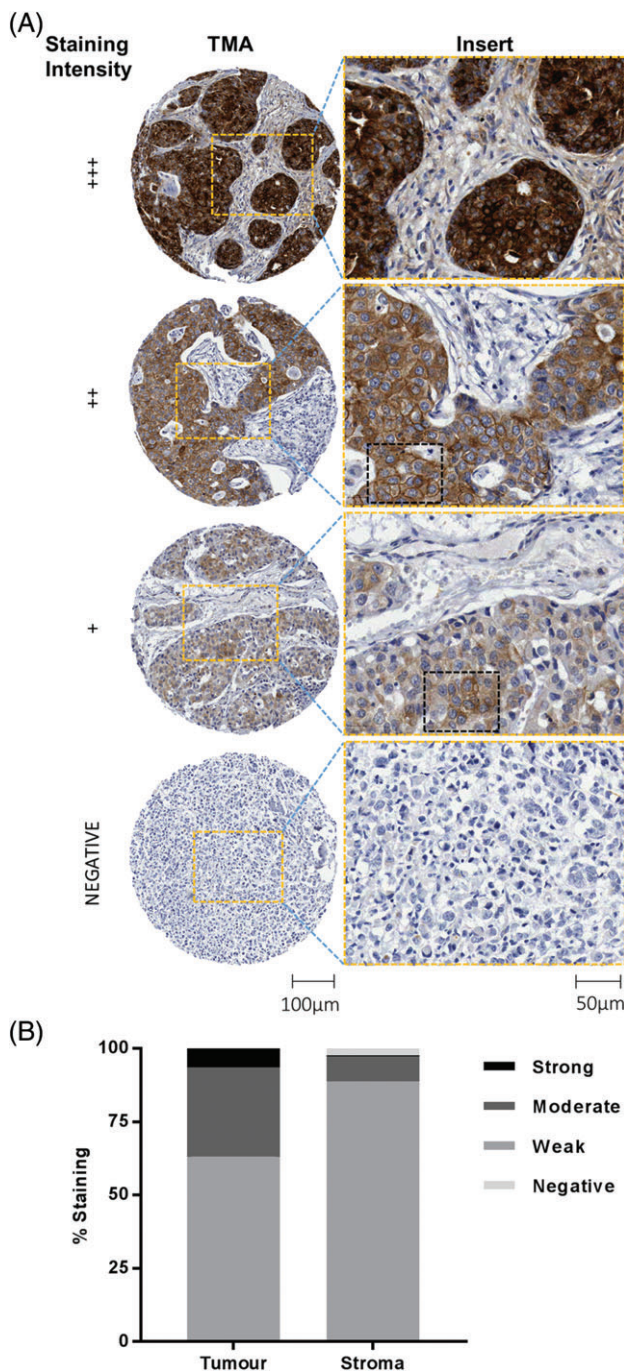


**Figure 1.** *STC2* overexpression in MBC. Significantly higher expression of *STC2* was seen in MBC ( $n = 12$ ) compared to FBC ( $n = 10$ ) (A), also implied by RT-qPCR analysis of three male and three female cases (B) and in cultured primary fibroblasts derived from male ( $n = 4$ ) and female ( $n = 3$ ) BC (C). While the number of MBC cases in the Oncomine™ analysis is low ( $n = 4$ , compared with 322 females), data mining showed higher expression of *STC2* in MBC versus FBC (D). Data on graphs are displayed as mean  $\pm$  SD, except (D) where data are displayed as median, 90th percentile and 10th percentile (minimum and maximum values also shown). M, male; F, female.

high levels of overall STC2, in tumour cells or stroma, had significantly longer DFS (Log-rank;  $p = 0.035$ ,  $p = 0.017$ ,  $p = 0.03$ , respectively). For cases where tumour cells had high levels of STC2, OS tended to be longer although this was not significant (Log-rank;  $p = 0.069$ ). There was no significant difference in OS for cases with high compared to low levels of STC2 total or in stroma (Log-rank;  $p = 0.23$ ,  $p = 0.65$ , respectively).

Multivariate analysis (with covariates patient age at diagnosis, node staging, tumour size, ER, and PR status) showed that total STC2 expression was an independent prognostic factor for DFS but not OS (Cox regression analysis; respectively  $p = 0.018$ ,  $p = 0.911$ ). Similarly, high STC2 in tumour cells was an independent prognostic factor for DFS, but not OS (Cox





**Figure 2.** Representative images of STC2 staining in MBC TMAs. The top panel shows examples of the various staining intensities in individual TMA cores, with higher magnification areas shown in the yellow outlined inserts. Black outlined inserts indicate foci of plasma membrane staining. The graph below shows the % of TMA samples which were categorised into each 'intensity' group (where the majority of tumour cells had at least the given intensity). +, weak, ++, medium staining, +++, strong staining. Images scanned at x20 objective magnification.

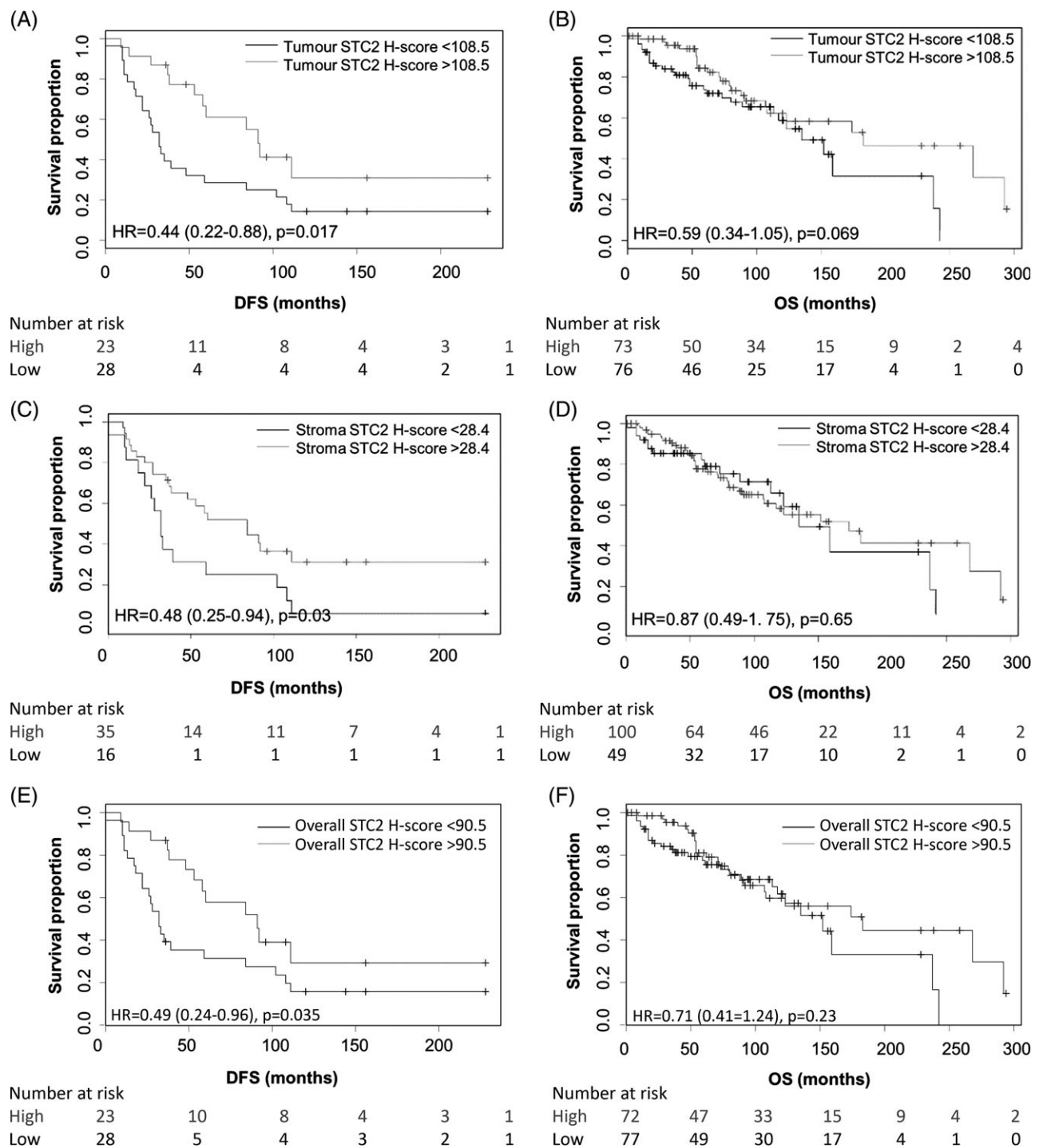
regression analysis; respectively,  $p = 0.015$ ,  $p = 0.822$ ). Patients with tumours containing stroma with high STC2 tended to have longer DFS, however, this was not significant (Cox regression analysis;  $p = 0.218$ ). Nor was there any relationship between stroma STC2 levels and OS (Cox regression analysis;  $p = 0.65$ ). Data are summarised in Table 2, with significant values in bold underline.

## Discussion

A number of studies are beginning to show that STC2 expression is a favourable prognostic factor in BC; however, it has not been studied previously in the context of MBC. With growing recognition that male and female BC may not be identical, there is increasing interest in elucidating the biology of MBC, to assist in defining indicators of survival. The key findings in this study were elevated expression of *STC2* RNA in male versus female BC and that both total STC2 protein and its expression in tumour cells was an independent predictor of patient survival in MBC.

Using cell line models, it has been suggested that the association between *STC2* expression and favourable outcome may be a result of its ability to repress invasive behaviour [25]. Hou *et al* [25] found enhanced migration, motility, and expression of the transcription factors Slug and Twist in BC cell lines where *STC2* was silenced, which following radiation were also more anti-apoptotic compared to non-silenced control cells. Similarly, Raulic *et al* [5] noted a reduction in cell motility when BC cell lines were stably transfected with *STC2*, as well as decreased cell viability after serum withdrawal and reduced proliferation. This finding may be unique to BC as, in other cancers, including neuroblastoma [26], lung [16], ovarian [17], and gastric cancer [9], *STC2* expression has been reported to promote metastasis and is thought to be a poor prognostic factor. These seemingly opposing roles of STC2 again indicate its ability to mediate its effects through different signaling pathways dependent on the cellular context, possibly through dysregulation of calcium and phosphate dependent signaling [25].

In a study of 72 paired primary and metastatic BCs [7], STC2 expression was significantly higher in primary tumours that showed late relapse, leading the authors to suggest that STC2 may be involved in tumour dormancy. This is of particular interest in BC, a disease known for its tendency to recur many years after a patient has been in remission. Formation of distant metastases is believed to be an early event in BC



**Figure 3.** Kaplan-Meier survival curves showing impact of STC2 staining H-score in tumour and stroma on patient prognosis. High STC2 H-scores in tumour cells (A), stroma (C) and total (E) were associated with longer DFS ( $p = 0.017$ ,  $p = 0.03$ ,  $p = 0.035$ , respectively), but had no significant impact on OS for tumour (B), stroma (D) or total (F) ( $p = 0.069$ ,  $p = 0.65$ ,  $p = 0.23$ ). Grey line, high STC2 H-score; black line, low STC2 H-score, Log-rank test. Cases were dichotomised by STC2 H-score: H-score cut-off point was 108.5 for tumour cells (DFS  $n = 28$  low,  $n = 23$  high; OS  $n = 76$  low,  $n = 73$  high); 28.4 for stroma (DFS  $n = 16$  low,  $n = 35$  high; OS  $n = 49$  low,  $n = 100$  high) and 90.5 for total staining (DFS  $n = 28$  low,  $n = 23$  high; OS  $n = 77$  low,  $n = 72$  high). HR, hazard ratio, followed by confidence intervals shown in brackets.

Table 2. Multivariate analysis of STC2 expression in MBC

Variable	DFS (Total)		OS (Total)		DFS (Tumour)		OS (Tumour)		DFS (Stroma)		OS (Stroma)	
	p	HR (CI)	p	HR (CI)	p	HR (CI)	p	HR (CI)	p	HR (CI)	p	HR (CI)
Age	0.155	1.054 (0.98–1.133)	0.002	1.09 (1.033–1.15)	0.142	1.058 (0.981–1.14)	0.002	1.09 (1.034–1.15)	0.202	1.04 (0.979–1.104)	0.003	1.088 (1.03–1.15)
Tumour size	0.704	1.015 (0.938–1.099)	0.059	1.038 (0.999–1.079)	0.778	1.011 (0.935–1.095)	0.057	1.038 (0.999–1.079)	0.447	1.029 (0.956–1.107)	0.08	1.036 (0.996–1.078)
ER	0.133	0.646 (0.366–1.142)	0.069	0.75 (0.55–1.023)	0.148	0.656 (0.37–1.162)	0.071	0.751 (0.55–1.025)	0.075	0.619 (0.366–1.049)	0.089	0.759 (0.552–1.043)
PR	0.11	0.819 (0.641–1.046)	0.155	0.897 (0.772–1.042)	0.101	0.813 (0.636–1.041)	0.157	0.897 (0.772–1.043)	0.192	0.87 (0.705–1.073)	0.147	0.896 (0.771–1.04)
Node staging	0.068	2.153 (0.946–4.897)	0.671	1.123 (0.657–1.92)	0.07	2.145 (0.939–4.898)	0.689	1.114 (0.656–1.892)	0.052	2.359 (0.992–5.613)	0.557	1.176 (0.686–2.016)
STC2 (Total)	0.018	0.983 (0.97–0.997)	0.911	1 (0.993–1.007)								
STC2 (tumour)	-	-	-	-	0.015	0.984 (0.972–0.997)	0.822	0.999 (0.992–1.006)	-	-	-	-
STC2 (stroma)	-	-	-	-	-	-	-	-	0.218	0.988 (0.97–1.007)	0.704	1.002 (0.992–1.012)

CI, 95% confidence interval; DFS, disease-free survival; HR, hazard ratio; OS, overall survival. Significant values are in bold underline.

[27], but it is not fully understood why secondary cancer arises in only a subgroup of patients. Both this study and our data suggest that low-tumour levels of STC2 may have potential as a biomarker to identify a subgroup of patients at risk of early relapse in BC.

Previously, STC2 has only been evaluated in the context of its expression in tumour cells. Here, we noted that STC2 was found not only in the tumour cells but also in stroma. Univariate analysis showed that patients with tumours with STC2 in both tumour and stroma had significantly longer DFS. As STC2 is a secreted glycoprotein [5], with the secreted form of STC2 reported to be the most abundant in some tissues [28], it is difficult to confirm whether it is produced mainly in the tumour cells or in stroma. Our RT-qPCR data support the hypothesis that it is predominantly produced by the tumour cells, showing approximately four-fold higher expression in frozen tissue containing both tumour and stroma cells, compared to expression in cultured tumour-associated fibroblasts. However, these data were not directly comparable; the fibroblasts used in this study were not derived from the tumours used for our original transcriptomic screen or the RT-qPCR validation used here, and it was not possible to test *STC2* expression in tumour cells isolated from BC. While efforts to establish tumour epithelial cell cultures from male BC have been fruitless thus far, we were able to successfully generate tumour-associated fibroblasts. To our knowledge this is the first time this approach has been used experimentally and offers a new angle to study male BC.

*STC2* expression appeared higher in MBC than in FBC and this was corroborated through interrogation of Oncomine™. For the transcriptomic part of our study, we acknowledge the number of cases of male BC available was low. However, this is not unusual when studying a rarer cancer type. This is also true of publically accessible data mining platforms such as Oncomine™, which also have very small numbers of male BC, with the largest comparative dataset we could analyse from this having only four male cases. Nevertheless, in other cancers (lung, renal, leukaemia, and colorectal), no gender-specific differences were identified in *STC2* expression (data not shown).

It has been proposed that high expression of Stanniocalcins in primary BC may predict late BC recurrence, with both STC1 and STC2 implicated [7]. While this work was under review, expression of STC1 but not STC2 in the primary tumour was predictive of late recurrence in a large cohort of Danish BCs [29]. Taken together, at least in BC, this adds weight to the notion that STC2 appears to be a good prognostic factor for both genders, following observations in



FBC, where elevated *STC2* expression was associated with longer OS and DFS [15,18,19,30]. However, in our study, there was a reduction in its significance on multivariate compared to univariate analysis. This might be explained by the fact that we were unable to obtain complete clinicopathological data from some centers that contributed cases for our TMAs; as some of this was necessary for multivariate analysis, a note of caution is warranted.

It has been additionally reported that *STC2* is associated with ER+ FBC [13], supported by our findings that fibroblasts from ER+ MBC, or FBC expressed higher levels of *STC2* compared to those from ER–breast tumours. As exemplified in the two largest reported studies on MBC, which examined thousands of patients, ER expression is very common in MBC [3,31], hence it is not surprising to see the same association.

In summary, while overexpressed in male compared to female BC, *STC2* appears to be a good prognostic factor, irrespective of gender.

## Acknowledgements

This study was supported by Yorkshire Cancer Research (grant L378) and Breast Cancer Now (TBLEE2017). We are grateful to the Breast Cancer Now Tissue Bank and the Male Breast Cancer Consortium who kindly provided cases for this work.

## Author contributions statement

VS conceived experiments. CCG, MPH, AC, and SSR carried out experiments. AD, VS, and CCG carried out data analysis. SSR and SJ provided clinical data. JLJ, GC, LBJ, RK, ADB, MM, EP, JK, AMS, and AMH provided patient material. CCG and VS wrote the manuscript. All authors read and approved the final manuscript.

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